

material is a single molecular species, rather than a mixture of the native and slightly modified pigments.

Note added in proof: Since this account was written we have observed regenerations of 85 per cent in purified cattle rhodopsin, flooded with retinene₁, and bleached for 1 minute in intense light.

¹¹ Wald, G., *Documenta Ophthalm.*, **3**, 94 (1949).

¹² Shantz, E. M., Embree, N. D., Hodge, H. C., and Wills, J. H., *J. Biol. Chem.*, **163**, 455 (1946).

¹³ Millard, E. B., and McCann, W. S., *J. Applied Physiol.*, **1**, 807 (1949).

¹⁴ I am greatly indebted for this preparation to Dr. Edgar M. Shantz of the Research Laboratories of Distillation Products, Inc., of Rochester, New York.

¹⁵ Wald, G., Meyerhof Festschrift, *Biochem. et Biophys. Acta*, **4**, 215 (1950).

Based on a reaction discovered by Morton, R. A., Salah, M. K., and Stubbs, A. L., *Biochem. J.*, **40**, Proc. lix (1946).

¹⁶ Negelein, E., and Wulff, H.-J., *Biochem. Z.*, **293**, 351 (1937). Lutwak-Mann, C., *Biochem. J.*, **32**, 1364 (1938). We owe the basic information on aldehyde fixation to the studies of Carl Neuberg and his coworkers [cf. Neuberg, C., and Kobel, M., *Biochem. Z.*, **188**, 211 (1927)].

¹⁷ Wald, G., and Hubbard, R., *Proc. Natl. Acad. Sci.*, **36**, 92 102 (1950).

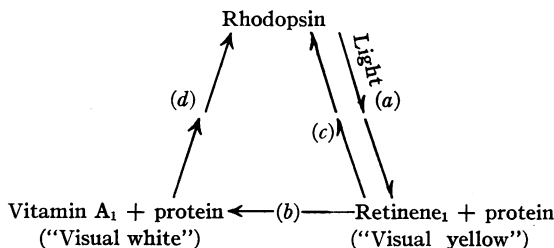
THE SYNTHESIS OF RHODOPSIN FROM VITAMIN A₁

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A number of years ago it was shown that in the rods of the retina, the light-sensitive pigment rhodopsin takes part in a cycle of the form:¹



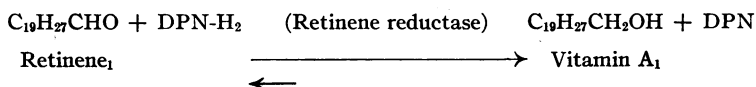
Some progress has been made recently with the chemistry of all these reactions. (a) In the bleaching of rhodopsin to retinene₁, the light reaction has been isolated and the orange intermediates, lumi- and meta-rhodopsin, characterized.² (b) Dihydrocozymase (DPN-H₂) has been identified as the coenzyme for the reduction of retinene₁ to vitamin A₁ by retinene reductase.³ (c) Rhodopsin has been synthesized from rhodopsin-protein and retinene₁, the latter prepared by oxidizing crystalline vitamin A₁ with manganese dioxide.⁴

There remains reaction (*d*), the retinal synthesis of rhodopsin from vitamin A₁. We can identify this with the process which Kühne long ago called "neogenesis," describing it as the formation of rhodopsin from colorless precursors. It occurs appreciably only in the intact eye, and Kühne believed that it requires the cooperation of the pigment epithelium. Since he failed to see any evidence of orange or yellow intermediates in the course of this process, Kühne concluded that it does not retrace the path by which rhodopsin bleaches.⁵ Indeed no direct evidence of a reversion of vitamin A₁ to retinene₁ can be found ordinarily in the isolated retina.

In the present paper, however, it is shown that isolated retinas, retinal homogenates and aqueous extracts of the retina can all form a little rhodopsin from vitamin A₁. When retinal homogenates are suitably supplemented, they synthesize almost half as much rhodopsin as is regenerated in the living eye during dark adaptation. It is shown also that one mechanism by which the retina can perform this synthesis—though perhaps not the only one—goes over retinene₁, and so retraces at least in part the path by which rhodopsin is bleached.

In the recent synthesis of rhodopsin from crystalline vitamin A₁ mentioned above, the first step—the oxidation of vitamin A₁ to retinene₁ (vitamin A₁ aldehyde)—was carried out, not by a retinal reaction, but by chromatographic oxidation on manganese dioxide. Retinene₁ prepared in this way condenses spontaneously with rhodopsin-protein to form rhodopsin.⁴ The success of this procedure suggested that this might be a route for the synthesis of rhodopsin from vitamin A₁ in the retina.

The retina contains a potential mechanism for oxidizing vitamin A₁ to retinene₁ in the retinene reductase system. Under equilibrium conditions, this system primarily *reduces* retinene₁, transferring two hydrogen atoms to it from dihydro-cozymase:



Similar DPN-enzyme systems in yeast and animal tissues reduce a variety of aldehydes to the corresponding alcohols.⁶ All of them are potentially reversible, but always their equilibria lie far over toward the side of reduction. To demonstrate their reversibility it is common practice to "trap" the aldehyde as fast as it is formed, with one of a number of aldehyde-binding reagents. In this way the system is kept from coming to equilibrium, and is driven continuously in the oxidative direction.

By this means one can also oxidize vitamin A₁ to retinene₁ in a retinal preparation. To fix the aldehyde, we have used 0.1 *M* hydroxylamine, NH₂OH. This couples spontaneously with retinene₁ to form a pale yellow product—presumably retinene₁ oxime—with an absorption maximum at

about 355 $m\mu$ in hexane and 363 $m\mu$ in aqueous solution. The extinction of retinene₁ oxime at its maximum is about 1.4 times that of the free aldehyde.

Dark adapted frog retinas (*Rana pipiens*) were isolated, and were bleached in bright light to colorlessness. In this state all their rhodopsin had been converted to vitamin A₁ and protein. They were then homogenized, suspended in neutral phosphate buffer and incubated at room temperature. The untreated homogenate, like the intact retina, does not form observable amounts of retinene₁, nor does it do so on adding DPN. In the presence of hydroxylamine, however, the untreated homogenate oxidizes an appreciable fraction of its vitamin A₁ to retinene₁; and the yield is greatly increased on adding cozymase.

These observations are illustrated in table 1. A homogenate of 16 frog retinas was divided into 3 equal portions. One was extracted at once with hexane (*a*). The other portions were incubated for 2 hours, one with hydroxylamine (*b*), the other with both hydroxylamine and DPN (*c*), and

TABLE 1

OXIDATION OF VITAMIN A₁ TO RETINENE₁ BY A FROG RETINAL HOMOGENATE, INCUBATED FOR 2 HOURS AT 23°C., WITH AND WITHOUT HYDROXYLAMINE AND DPN

The homogenate initially contained vitamin A₁ alone. The table shows the absorption maxima (λ_{max}) of hexane extracts of the homogenate, and the proportions of vitamin A₁ and retinene₁ which they contained. The homogenate was suspended in *M*/15 phosphate buffer, pH 6.84, and this buffer was also added in the amounts shown.

SAMPLE	RETINAL HOMOGENATE, ML.	BUFFER, ML.	HYDROXYL- AMINE (0.2 <i>M</i>), ML.	DPN, MG.	λ_{max} , $m\mu$	VITAMIN A ₁ TRANSFORMED TO RETINENE ₁ , %
<i>a</i>	0.4	1.6	322	0
<i>b</i>	0.4	0.6	1.0	...	327	28
<i>c</i>	0.4	0.6	1.0	2.0	335	49

were then extracted with hexane. The absorption spectra of these extracts were measured. As shown in table 1, their absorption maxima (λ_{max}) shifted from the initial position characteristic of vitamin A₁ (*a*), about 5 $m\mu$ toward the red on incubation with hydroxylamine (*b*), and about 13 $m\mu$ toward the red on incubation with both this reagent and DPN (*c*). From this shift of spectrum it could be computed that in (*b*) the final mixture of retinene₁ and vitamin A₁ contained about 28 per cent retinene₁, in (*c*) about 49 per cent retinene₁.

Another experiment is shown in figure 1. Dark adapted retinas were bleached to colorlessness, homogenized with neutral phosphate buffer and 3 equal portions of homogenate were incubated at room temperature with hydroxylamine and DPN. In (*a*) the reaction was stopped at once by adding methyl alcohol to a concentration of 60 per cent; in (*b*) it was stopped after 1 hour, in (*c*) after 2 hours. All three preparations were ex-

tracted with hexane. The absorption spectra of these extracts are shown in the figure. Vitamin A₁ alone was present initially (*a*); about 38 per cent had been converted to retinene₁ in one hour (*b*); and about 51 per cent in two hours (*c*). The differences between the initial and later spectra (*b* - *a*, *c* - *a*) show that the absorption had risen in the region of the retinene₁ maximum (positive differences) and fallen in the region of the vitamin A₁ maximum (negative differences).

It is evident from these data that in the presence of an aldehyde-fixative, retinal preparations can oxidize vitamin A₁ to retinene₁. This process is aided by supplementation with DPN, the coenzyme of the retinene reductase system. The added DPN apparently replenishes the supply of coenzyme present in the retina initially, but rapidly destroyed by a nucleotidase after homogenization.³

The retina normally possesses a specific process which binds retinene₁, its condensation with rhodopsin-protein to form rhodopsin. Like all proper trapping reactions, there is an energy-yielding process. It is not nearly so efficient as the binding of retinene₁ by hydroxylamine, and is therefore nearly completely blocked in the presence of this reagent.⁴ In the retina, however, rhodopsin-protein may substitute physiologically for hydroxylamine, and may drive a

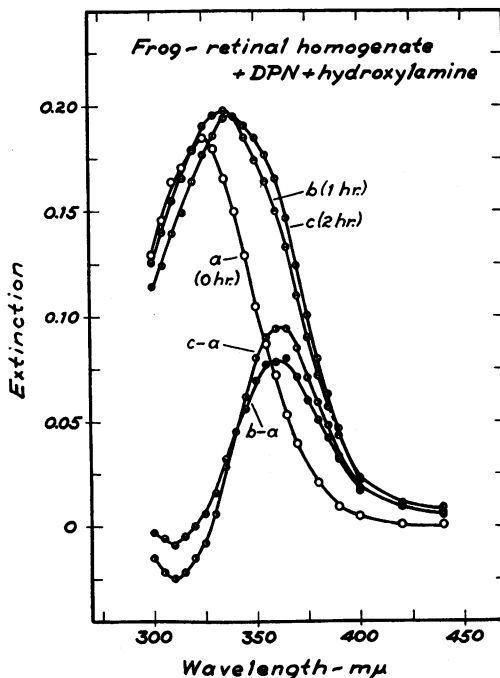


FIGURE 1

Oxidation of vitamin A₁ to retinene₁ in a homogenate of frog retinas containing 0.1 *M* hydroxylamine and 2 mg. DPN per ml. In (*a*) the reaction was stopped at once by adding methanol, in (*b*) after 1 hour of incubation at 23°C., in (*c*) after 2 hours incubation. pH 6.8. Absorption spectra of hexane extracts of these preparations are shown (*a*, *b*, *c*). The initial spectrum (*a*) is that of vitamin A₁; but this is displaced toward the red as the reaction proceeds. The changes in absorption (*b* - *a*, *c* - *a*) show a rise in the region of the retinene₁ maximum (positive differences), a fall in the region of the vitamin A₁ maximum (negative differences). From these data it is computed that 38 per cent of the vitamin A₁ initially present had been oxidized to retinene₁ in 1 hour, 51 per cent in 2 hours.

continuous oxidation of vitamin A₁ to retinene₁, by removing retinene₁ to form rhodopsin.

The difficulty with this idea is that the isolated retina, bleached to colorlessness, does not regenerate rhodopsin efficiently from vitamin A₁, though it contains all the components of the system described above—retinene reductase, cozymase and rhodopsin-protein. It is primarily this behavior of the isolated retina that caused Kühne and later workers to conclude that the synthesis of rhodopsin from colorless precursors requires new materials, and involves something more than the simple reversal of bleaching. This inadequacy of the isolated retina constitutes the primary problem with which our further experiments are concerned.

Frog retinas, isolated in the dark adapted condition, and bleached in the light to colorlessness, contain vitamin A₁ as their only carotenoid. If they are replaced in the dark for several hours, they still appear colorless, or at most faint pink. If, after incubation in the dark, they are extracted with 2 per cent aqueous digitonin, the extract is found to contain a small amount of rhodopsin. This is conveniently revealed by measuring the absorption spectrum of the extract in darkness, then again after bleaching in the light. The difference in absorption spectrum before and after bleaching—the so-called “difference spectrum”—is characteristic of rhodopsin.

The yield of rhodopsin in such an experiment can be estimated by extracting dark adapted frog retinas with digitonin by the same procedure. The difference spectra of such extracts show that on the average each retina contributes an amount of rhodopsin having an extinction at 500 mμ of about 0.08 in 1 ml. of extract, measured in a layer 1 cm. in depth.

On this basis the intact bleached retina replaced in the dark regenerates from vitamin A₁ about 10 per cent of its potential content of rhodopsin. The yield is not increased by adding DPN. This in itself is not significant, since DPN probably does not penetrate the intact tissue.

A homogenate of bleached retinas regenerates about as much rhodopsin in the dark as do intact retinas. When DPN is added to such a homogenate, however, the yield of rhodopsin is approximately doubled.

Such an experiment is shown in figure 2. Sixteen retinas had been bleached in bright light for 45 minutes, and were wholly colorless. They were homogenized with neutral phosphate buffer, and divided into three equal portions. The first of these, extracted at once with hexane, showed the presence of vitamin A₁ alone (*A*). The other two portions were incubated in the dark at room temperature for 9 hours, one untreated (*B*), the other with DPN added (*C*). They were then extracted with digitonin, and the spectra of the extracts measured before and after bleaching. These difference spectra are shown at the left in figure 2. The solid residues from these extractions were reextracted with hexane; the spectra of these extracts are shown at the right.

The hexane extracts show that vitamin A₁ alone is present, both in the original homogenate and in the portions incubated in darkness. The difference spectra at the left are characteristic of rhodopsin. They show that the untreated homogenate regenerated about 10 per cent of rhodopsin, while the homogenate incubated with DPN yielded almost twice this amount, about 18 per cent (cf. table 2).

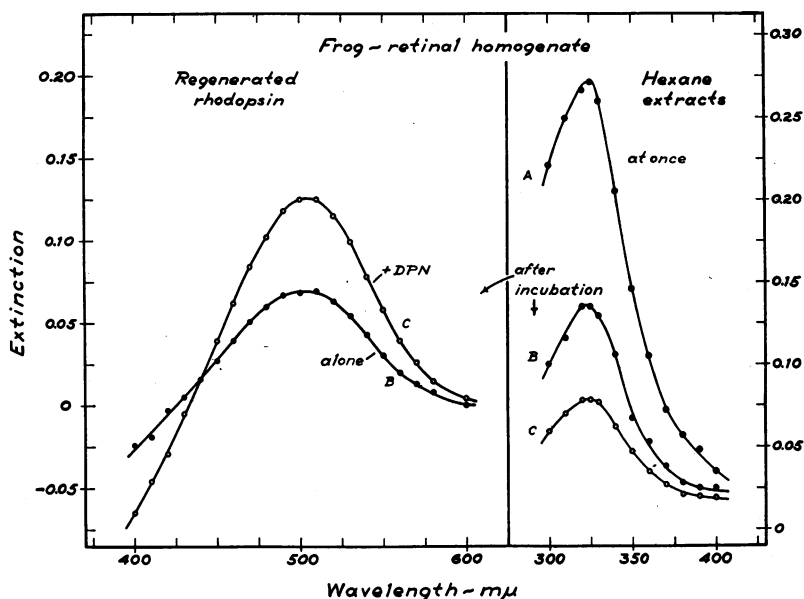


FIGURE 2

Synthesis of rhodopsin from vitamin A₁ in a retinal homogenate, incubated in the dark 9 hours at 23°C., with and without added cozymase (DPN, 4 mg. per ml.). The data at the left show difference spectra of digitonin extracts of these preparations—their differences in absorption before and after exposure to light. They are characteristic of rhodopsin, and show that supplementation with DPN approximately doubles the yield of this pigment. The absorption spectra at the right are of hexane extracts (A) of the original homogenate, and (B) and (C) of the residues of the incubated homogenate after extraction with digitonin. They show that vitamin A₁ is the only carotenoid present initially and at the end of the reaction.

The effectiveness of adding DPN in this synthesis suggests that it was accomplished through the oxidation of vitamin A₁ to retinene₁ by the retinene reductase system, retinene₁ being trapped by rhodopsin-protein as rapidly as formed. The data show that in the course of this process no free retinene₁ accumulates. Kühne's failure to see yellow intermediates during the "neogenesis" of rhodopsin is thus confirmed. Yet this is no assurance that retinene₁ is not an intermediate in the reaction.

We have already noted Kühne's insistence upon the co-operation of the pigment epithelium in "neogenesis." We find that when a homogenate of the pigment layers of the eye—pigment epithelium and choroid—is added to a retinal homogenate, the synthesis of rhodopsin from vitamin A₁ is approximately doubled. When both DPN and pigment layers are added to the retinal homogenate, the yield of rhodopsin is again doubled; it now approaches 40 per cent.

Such an experiment is shown in figure 3. Sixteen frog retinas were isolated in the dark, and were bleached to colorlessness in the light for 1 hour. They were homogenized in neutral phosphate buffer and the homogenate was divided into 4 equal portions. To one of these, the homogenate of the pigment layers from 12 eyes was added together with 4 mg. of DPN (*d*). To two other portions, 2 and 4 mg. of DPN alone were added (*b*, *c*). The

TABLE 2

PER CENT REGENERATION OF RHODOPSIN IN RETINAL HOMOGENATES, INCUBATED IN THE DARK FOR 8-9 HOURS UNDER VARIOUS CONDITIONS.

All homogenates were suspended in neutral phosphate buffer, final pH about 6.8. 23°C. The per cent regeneration represents the extinction at 500 m μ of the regenerated rhodopsin as a fraction of the average extinction at 500 m μ of rhodopsin from an equal number of dark adapted retinas.

EXPERIMENT	DESCRIPTION	PER CENT REGENERATION
I. (Fig. 2) (<i>a</i>)	Homogenate alone	10.0
	(<i>b</i>) Homogenate + DPN	18.3
II. (Fig. 3) (<i>a</i>)	Homogenate + DPN	17.9
	(<i>b</i>) Homogenate + DPN + pigment layer homogenate	30.3
III.	Homogenate + DPN	25.4
IV. (<i>a</i>)	Homogenate + pigment layer homogenate	20.3
	(<i>b</i>) Homogenate + pigment layer homogenate + DPN	42.0

fourth portion (*a*) was extracted immediately with hexane; so also was a control portion of pigment layers (*e*). Homogenates *b*, *c* and *d* were incubated for 8 hours in the dark, and were then extracted with digitonin solution. The difference spectra of these extracts are shown at the left of figure 3. After extraction with digitonin, the solid residues were reextracted with hexane; the absorption spectra of these and the control hexane extracts are shown at the right of the figure.

It is clear from figure 3 that the addition of 2 mg. of DPN to a total of 0.7 ml. of reaction mixture produces a maximal effect on the regeneration of rhodopsin (*b*). Doubling this amount does not improve the yield further (*c*). On the other hand the addition of both DPN and homogenized pigment layers approximately doubles the yield of rhodopsin (*d*) (cf. table 2). The pigment epithelium therefore must add something other than DPN to the reacting system.

The spectra of carotenoid extracts at the right of figure 3 show that vitamin A₁ alone is present in the retinal homogenates, vitamin A₁ and xanthophyll in the homogenates containing pigment layers. In no case is any retinene₁ apparent, either in the tissues extracted at once or in those incubated in darkness.

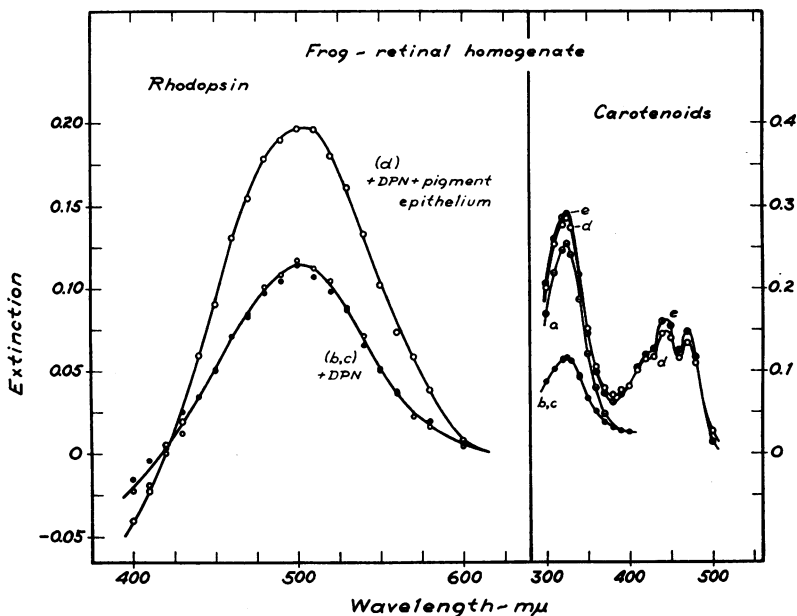


FIGURE 3

Synthesis of rhodopsin from vitamin A₁ in a retinal homogenate, incubated in the dark 8 hours at 23°C., with (b) 2 mg. and (c) 4 mg. DPN added; and with (d) 4 mg. DPN and pigment layer homogenate added. The data at the left show the difference spectra of digitonin extracts of these preparations. They are characteristic of rhodopsin, and show that supplementation with 2 mg. DPN is as effective as 4 mg. DPN (b, c); but that the addition of pigment layer homogenate approximately doubles the yield of rhodopsin. The absorption spectra at the right are of hexane extracts of the solid residues of these preparations, and of control portions of the original retinal homogenate (a) and of the pigment layer homogenate (e). They show that the retinal preparations contain vitamin A₁, the pigment layer preparations vitamin A₁ and xanthophyll as their only carotenoids at the beginning and end of the reaction. Vitamin A₁ is responsible for the single absorption band in the ultra-violet at about 325 mμ, xanthophyll for the complex spectrum in the visible region.

The yields of rhodopsin obtained in retinal homogenates by all the types of treatment described above are summarized in table 2. We have, however, also observed the synthesis of rhodopsin from vitamin A₁ in aqueous solution.

An aqueous digitonin extract of bleached frog retinas contains rhodopsin-protein and retinene reductase. If freshly prepared, it also contains cozymase, though this is destroyed within a few hours by the nucleotidase which is present.³ The addition of a high concentration of vitamin A₁ to such an extract might be expected to force the production of some retinene₁, and from this could induce the synthesis of some rhodopsin.

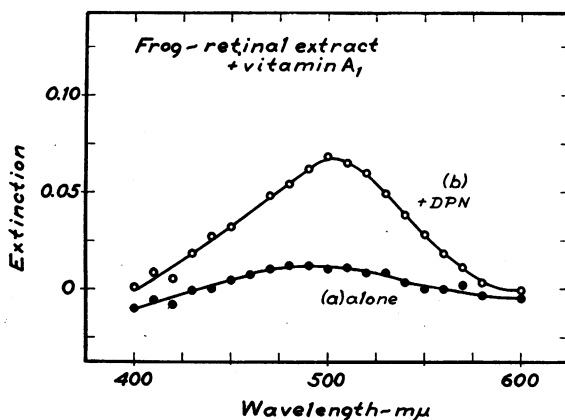


FIGURE 4

Synthesis of rhodopsin from vitamin A₁ in an aqueous extract of frog retinas. The retinas had been bleached to colorlessness, extracted with 2 per cent aqueous digitonin, and to this crystalline vitamin A₁ in digitonin was added. Half of this preparation was incubated in the dark overnight at 23°C. without further treatment (a), the other half with 4 mg. DPN added per ml. of extract (b). The data show the difference spectra of the final solutions. The solution without added DPN had synthesized a scarcely perceptible amount of rhodopsin; that supplemented with DPN had regenerated about 10 per cent as much rhodopsin as was present originally in the dark adapted retinas from which it was prepared.

Such an experiment is shown in figure 4. Twelve dark adapted frog retinas were isolated, bleached to colorlessness, homogenized and the homogenate extracted for 3 hours with digitonin. The extract was cleared by centrifuging at high speed, and to it a clear solution of crystalline vitamin A₁ in digitonin was added. The mixture was divided into halves, and to one portion 3.5 mg. of DPN was added. Both portions were left in the dark for 12 hours, and then their difference spectra were measured. These are shown in the figure. The solution to which no DPN had been added (a) regenerated an almost negligible amount of rhodopsin. That supplemented with DPN (b) had regenerated about 10 per cent as much rhodopsin as was

present originally in the dark adapted retinas from which the solution was prepared.

In summary, therefore, we have found that rhodopsin is synthesized from vitamin A₁ in intact retinas and retinal homogenates, with yields of about 10 per cent. In retinal homogenates supplemented with either DPN or pigment layer homogenate, the yields are about 20 per cent; and in homogenates supplemented with both DPN and pigment layers they rise to about 40 per cent. Aqueous extracts of retina, to which DPN and vitamin A₁ have been added, also synthesize rhodopsin with a yield of about 10 per cent.

The mechanism of rhodopsin synthesis in these preparations is still uncertain. We know one possible mechanism, the oxidation of vitamin A₁ to retinene₁ by the retinene reductase system, coupled with the condensation of retinene₁ with rhodopsin-protein to form rhodopsin. Yet this apparatus, as we find it in the isolated retina, appears unable to account for more than a small fraction of the rhodopsin formed in the intact eye.

It seems reasonably clear that DPN stimulates the synthesis of rhodopsin in our preparations by supplying coenzyme to the retinene reductase system. Pigment layer homogenate may act upon the same system, by supplying respiratory factors which drive it in the oxidative direction. In this regard it is perhaps significant that riboflavine, the common oxidant of DPN-H₂ in cellular respiration, is present in high concentration in the pigment epithelium, though little is found in the retina.⁷

We think it almost certain that some rhodopsin is synthesized through the intermediate oxidation of vitamin A₁ to retinene₁ by retinene reductase. It is conceivable that by coupling with respiratory mechanisms, the efficiency of the retinene reductase system is so greatly increased that it can account for the whole synthesis of rhodopsin from vitamin A₁. On the other hand alternative pathways for this process may exist, and may even be of major importance. These problems are being investigated further.

Summary.—Intact frog retinas and retinal homogenates can synthesize rhodopsin from vitamin A₁ in amounts about 10 per cent as great as are formed during dark adaptation *in vivo*. When either cozymase (DPN) or a homogenate of the pigment layers of the eye—pigment epithelium and choroid—are added to a retinal homogenate, the yield of rhodopsin is approximately doubled; and by the addition of both DPN and pigment layer homogenate, the yield is doubled again, bringing it to about 40 per cent. Aqueous extracts of retina, supplemented with DPN and vitamin A₁, also synthesize rhodopsin with a yield of about 10 per cent.

The mechanism of this synthesis is not yet wholly understood. Retinal homogenates slowly oxidize vitamin A₁ to retinene₁ (vitamin A₁ aldehyde) in the presence of such an aldehyde-binding reagent as hydroxylamine. This process, like rhodopsin synthesis, is aided by supplementation with

DPN, the coenzyme of the retinene reductase system. Hydroxylamine apparently drives the retinene reductase system in the oxidative direction, by removing retinene₁ as fast as it is formed.

In the retina, rhodopsin-protein may act similarly, since it is known to bind retinene₁ by condensing with it spontaneously to form rhodopsin. This is almost surely one mechanism for the synthesis of rhodopsin from vitamin A₁. Yet the isolated retina, which contains all the components of this system, regenerates very little rhodopsin from vitamin A₁. Either the retinene reductase system operates much more efficiently in the whole eye than in the isolated retina, through the action of such auxiliary factors as may be added by the pigment epithelium; or some rhodopsin is synthesized from vitamin A₁ by alternative mechanisms still to be explored.

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ANTIBIOTIC SUBSTANCES FROM BASIDIOMYCETES. VI. AGROCYBE DURA*

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Communicated December 19, 1949

In a previous report¹ from this laboratory, *Agrocybe dura* (L386.10)² was found to evidence considerable activity against *Staphylococcus aureus* (H) and *Escherichia coli* when tested by the streak of disk methods. Culture liquids of this fungus were found to have some activity. Further investigation has resulted in additional information on the antibiotic properties of *Agrocybe dura*.

Antibiotic Material in Liquid Culture.—Culture liquids with antibacterial activity were produced by growing the fungus at 25°C. in 2800 ml. Fernbach flasks containing a corn steep medium, as previously described.³